THE CULTIVATION OF BACTERIA, AND THE CHOLERA BACILLUS.

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In order to be sure that a disease is caused by any germ, five conditions must be fulfilled. (1) It must be found in the blood or tissues of every one affected with the disease. (2) It must not be found in the body of persons not affected with the disease. (3) It must be cultivated outside of the body in such a way as to ensure its freedom from other forms, and for generations enough to be sure that it has been freed from any other poison contained in the body. (4) This pure culture must then be inoculated into the body of some other person, and the original disease must result. (5) The germ must be found in the last case. If any one of these steps is wanting, the proof of the causation of the disease is incomplete.

The detection of the germ in the body of the diseased person is, for the most part, comparatively easy, and is well enough understood; but if we may judge from much that has been written lately on the cholera bacillus, the second step is by no means so well known.

There are two methods of culture in use: the culture in fluids, and that on a firm soil. Culture in fluids has several disadvantages. In the first place, any part of the body to which air can gain access will contain several forms of bacteria, as in the case of pus from an ulcer, sputum, etc. It is impossible in fluid cultures to separate these forms and detect which one is the cause of mischief. But even if pure in the first place, it is difficult to keep cultures entirely free from contamination from germs contained in the air, and other sources. In fluid cultures, on account of the mobility of most forms, these impurities become thoroughly mixed with the original forms, and it is impossible to distinguish one from the other. Indeed, many forms are inimical to each other, and the intruders may de-

stroy the original forms and leave something entirely different in their place. Dr. Koch has, therefore, devised his method of cultivation on a fixed soil. Several substances are used for this purpose, but the most common are peptonized beef broth mixed with gelatine, and the potato. The bacteria are unable to move on these materials, and if an impurity finds its way into the culture it can be detected and removed. The preparation of peptonized gelatine is as follows: 250 grammes of fresh beef, as free as possible from fat, is chopped fine, placed in 500 grammes of distilled water, and allowed to stand over night upon ice. The mixture is then shaken repeatedly and pressed through a fine cloth; distilled water enough is added to make 400 cubic centimeters; to this, 4 grammes (1 per cent.) of "*Peptonum Siccum*," 2 grammes of common salt and 40 grammes of fine white gelatine are added. This mixture is allowed to macerate for about half an hour, and is then warmed in a water bath so as to melt the gelatine without precipitating the albumen. It is then carefully neutralized with a saturated solution of bicarbonate of soda, adding the solution, drop by drop, until blue litmus paper remains blue, but red paper shows a slight blue tinge. For the cholera bacillus this neutralization must be very carefully done, for the bacillus will grow only when the exact stage of neutralization is reached. In case the mixture has become too alkaline, a little lactic acid should be added. The mixture is then boiled from half an hour to an hour, in order to precipitate the albumen; filtered, and if not quite clear, boiled and filtered again. It should then be tested again to make sure that the reaction has not changed, which it is somewhat liable to do. By this cooking the color of the mixture is changed to a faint yellow. After boiling, the mixture is filtered into a microscopically clean vessel and again boiled until no cloudiness appears. It should then be clear and of a topaz-yellow color. The mixture may be preserved in test-tubes which have been stopped with cotton and steralized by heat. In laboratories it is usual to have an oven, which can be kept at a uniform high temperature, for this purpose. The temperature required to be maintained is from 160° to 180° centigrade, continued for an hour. Large numbers of test-tubes can be so steralized very conveniently. The oven is, however, by no means necessary. If the tube is cleaned

and a plug of cotton placed in the mouth, it can be easily steralized by heating in the flame of a Bunsen burner, or alcohol lamp; taking care, of course, in order to avoid burning the plug, to push it into the mouth of the tube a short distance with the points of forceps *which have been heated.* If the cotton is heated just enough to brown it slightly, it is sufficiently steralized. The test-tube itself is steralized if heated all over so that it feels warm when held close to the face. The tubes are then filled about one-third full with the peptonized gelatine. A pipette with a bulb in the middle, which has been steralized by passing through a flame, is convenient for this purpose. They are then placed in a vessel of water, first putting six or eight layers of muslin under them to prevent their breaking, and boiled a quarter of an hour, or more. The ebullition should not be rapid enough to bring the gelatine in contact with the cotton, or it will be difficult to withdraw the plug when desired. This boiling is repeated at intervals of from twelve to twenty-four hours, for five or six times; the reason of this repetition in boiling being that adult germs are destroyed by a temperature of boiling water, but many spores are not. By the first boiling, the adult forms that may have found their way into the gelatine, will be killed, but if any spores are present they may remain alive. In from twelve to twenty-four hours these spores will have begun to develop adult forms, which will be killed by the boiling before they have had time themselves to form spores. After five or six of these boilings it may be safely concluded that all the spores that are to develop at all, have done so, and that the mixture is steralized. As a further precaution, however, it is desirable to keep the tubes a few days before using, in order to see that no forms develop in them.

Having secured the steralized gelatine we are prepared to begin the cultures. These cultures are of two sorts: plate cultures and needle cultures. Plate cultures are for the purpose of securing the germ in a pure state. For this purpose there are provided a series of glass plates about four inches by six, and some benches made of strips of glass about six inches long and two inches wide, to the ends of which have been cemented strips of glass about one-quarter of an inch in thickness. In order to protect these plates from dust the laboratories are provided with flat and low glass shades, similar

to the cake covers used by confectioners, which fit into shallow glass vessels—these are called moist chambers. If these are not at hand, however, the confectioner's cake cover and a dinner plate, or even two soup plates, will do very well. A platinum wire about one and a half inches long, melted into the end of a glass rod, and a similar one bent into a loop at the end, and an ordinary glass stirring-rod are also necessary.

The bell glass is now washed out with a solution of corrosive sublimate (1 in 1000), and a layer of filter paper moistened with the same solution is placed in the bottom of the moist chamber. The plates are then passed through the flame of a Bunsen burner or an alcohol lamp, laid upon blotting paper on the table, and covered with the bell in order to cool. Three or four, or more, of the test-tubes containing the steralized gelatine are warmed sufficiently to melt the gelatine, and the cotton plugs are loosened to make sure of their easy removal. The platinum needle with the loop upon the end is passed through the flame and allowed to cool. The test-tube containing the gelatine is taken between the thumb and forefinger of the left hand with the palm turned obliquely upwards. In order to prevent dust falling into the open end, the tube is inclined as much as possible without the gelatine touching the cotton. A small flocculus of mucus from the cholera stool, about the size of a pin's head, is then taken up with the platinum wire; the plug of cotton is removed from the test-tube by the third and little fingers of the right hand, and the mucus is introduced into the gelatine, and thoroughly mixed by stirring with the needle. The plug is returned to the tube, and the tube is shaken to ensure the more perfect mixing of the whole. A little wetting of the cotton in this case does no great harm. Another tube of gelatine is then taken, prepared as before and placed between the first and second fingers, while the first tube is held between the thumb and first finger as before. The plugs are then removed from the tubes and placed respectively between the second and third, and third and fourth fingers of the left hand, taking care to touch only the upper part of the cotton. Five drops are then taken from the first tube, with the platinum wire, and placed in the second tube giving the wire a little shake each time to ensure the detachment of the drop. These drops are

also thoroughly mixed with the gelatine as in the former case. A third tube is prepared by taking, in the same way, five drops from the second tube. The gelatine in the tubes is allowed to cool to a point where it is just about to stiffen but is still fluid enough to flow, and is then poured out on the plates and, if necessary, spread around with a steralized glass rod, leaving a rim about half an inch wide at the edges. The plates are then piled, one over the other, upon the glass benches in the lower part of the moist chamber, first putting under each plate, upon its bench, an appropriate label. The object of this whole procedure is to spread the germs over a considerable surface by diluting the fluid in which they are held. They can be so spread that the individual germs will be separated from each other by an interval of half an inch or more, and the growth of each germ can be studied by itself, under the microscope, and the particular sort that is wanted can be fished out with a steralized platinum needle. In order to ensure the perfect freedom of the germs from any mixture, it is advisable to repeat the culture two or three times. When this has been done it is quite certain that the culture is pure. Minute directions for picking up these colonies are given in the laboratory. The needle is bent into the form of a small hook, about a millimeter in depth, before being heated. The plate is then placed upon the stage of the microscope, and a typical colony selected, a low power—an inch or three-quarters—being used. The little finger is rested upon the stage, the needle is brought into view over the colony, the point of the hook is then lowered into the colony and again raised perpendicularly upwards and removed.

Having the pure plate culture, we are in a condition to make the needle culture. This is done by picking up one of the colonies on the plate with the platinum needle and plunging it nearly to the bottom of the cold gelatine in one of the tubes, and studying its method of growth in this situation.

While the culture is going on in the gelatine, it is also desirable to study the growth of the forms in the "hanging drop." A slide which has been hollowed out to the depth of from one to one and a half millimeters is needed for this purpose. The slide is cleaned and passed through the flame. After cooling, a ring of vaseline is run around the edge of the exca-

vation. Too much vaseline must not be used or it will prove troublesome by running under. A cover is also cleaned and heated. A drop of the peptonized beef without the gelatine is placed in the middle of the cover, and a small portion of the cholera mucus is rubbed into the drop without spreading it. The cover is then quickly inverted over the chamber, being careful, of course, that the drop does not reach the sides of the cell: the process of growth may now be studied under high powers.

The culture upon potatoes is made in the following way. A potato that is soggy when boiled, and one as round and smooth and free from defects as possible, is selected. This potato is carefully cleaned with soap and water and a brush, and all defects are scraped away with a knife, with as little injury to the skin as possible. It is then soaked for an hour in a half per cent. solution of corrosive sublimate and then steamed for half an hour. The blade of a dissecting knife is passed through the flame, and allowed to cool with the blade projecting, edge upwards, over the side of a table. The left hand is then dipped in a solution of corrosive sublimate (one part in a thousand) and the potato is taken up with the thumb and finger of this hand. It must never be touched with the other hand. The knife is taken with the right hand and passed nearly through the potato, and withdrawn without separating the halves, leaving a small portion of the skin so that they will not fall apart. The potato is then laid down in the moist chamber, which has been prepared as in the former case, and covered with the bell glass. A little of the infected material is then taken up on the point of the knife; the halves of the potato are for the first time separated, and the material spread over one half, as butter is spread upon bread, taking pains to spread the material evenly and not to bring it quite to the edge. A bit taken from the first half is then spread upon the other half of the potato. The dilution may be carried on in the same way to a second or even a third potato.

The cholera bacillus was discovered by Koch in the stools of persons suffering from cholera. In a hundred cases of this disease it was found always present and always the same. In its living condition it resembles somewhat the ordinary bacterium termo, but instead of being straight, it is curved not far from a quarter of a circle;

often two or more are connected together, sometimes forming a semicircle when the concave surfaces point the same way; or an old-fashioned long S, when they point different ways. Occasionally they form a wavy line. When sown upon plates, the cholera bacilli grow rather slowly; the colonies which form here and there over the plate very soon acquire a notched outline which is characteristic. The color of the young colonies is a light yellowish red, and the individuals are strongly refractile, so that the whole appears coarsely granular and as though composed of fine bits of broken glass. The gelatine is liquefied into a funnel-shaped excavation, broader above and smaller below. After five or six days the gelatine on the plate is not all liquefied.

In the needle cultures the upper part of the track of the needle begins to liquefy in a funnel shape, and in a day or so an air-bubble appears at its top. The lower part of the track of the needle appears as a fine white thread. It takes from four to six weeks for the whole gelatine to become liquefied. No offensive gases are formed, the extent of odor being a slightly pungent smell, resembling urine.

The bacillus will grow upon potatoes only at a temperature of about 37° centigrade, forming a dark-brown layer.

The bacillus does not form spores, and is not found in the blood or in any organ of the body except the mucous membrane of the intestine. It does not grow in acid fluids, consequently persons with vigorous digestions are not liable to cholera. It is only persons whose digestion is temporarily arrested who are liable to the disease, as is shown in the history of epidemics.

Giving the bacilli to the lower animals by the mouth has not caused the disease, owing, no doubt, to the great activity of their digestions. It is well known that they are not subject to cholera, probably for this reason. Injections into the intestinal canal, however, have given rise to symptoms exactly resembling cholera, with large numbers of the bacilli in the stools and in the intestinal canal.

Several forms which resemble this bacillus have been described; for, of course, every crooked bacillus attracts attention now. The most noted of these is that discovered by Finkler and Prior in the stool of a patient with cholera morbus. This bacillus was described, as being exactly like the comma bacillus of Koch, and consequently

attracted a great deal of attention. It must be borne in mind that it is in no way characteristic of cholera morbus, being never found in the fresh stools. This form was discovered in an old stool that had been exposed to the air for fourteen days, and all the bacilli of this kind which are known, have descended from this sample. In the dried preparation on the slide these bacilli resemble Koch's bacilli, but are larger in every way, and especially thicker. In the living state this difference is much more plainly marked. But cultivation shows other and widely different characteristics. In plate culture the plates soon acquire a distinct, brownish yellow color in place of the yellowish red of Koch's bacillus. The colonies have a sharp outline and a *fine* granular appearance. In about twenty-four hours they are surrounded by a broad zone of liquefied gelatine. The colonies soon break up into an irregular plate and the culture gives rise to an exceedingly offensive odor. In three days, at the longest, the gelatine is all liquefied.

In needle cultures the gelatine begins to liquefy at once the whole length of the puncture, and in a day or two a large coarse depression is excavated in the gelatine, not so funnel shaped as in the case of Koch's bacillus, but many times larger, and wanting the bubble of air. In a few days the whole of the gelatine is liquefied. On potatoes, the Finkler-Prior bacillus grows luxuriantly at the ordinary temperature of the room, forming a nasty, slimy, greyish yellow coating with a whitish bounding zone, and rapidly eats into the substance of the potato. These differences seem to be quite sufficient to show that the two forms are not the same.

Two other sorts of comma bacilli have been described: one sometimes found in the mouth, and another in old cheese. The bacillus from the mouth, in spite of Klein's assertions, will not grow on the alkaline gelatine. The bacillus of cheese will grow on gelatine, but in a way different from the cholera bacillus, and is not pathogenic.

The proof that the bacillus is unlike any other form and is peculiar to cholera, seems conclusive. That it is the cause of the disease seems to me scarcely less so.

A practical conclusion or two, and I have done. The disease is not contagious as small-pox and measles are; it is only by the bacillus gaining access to the intestinal canal that the disease is caused.

If this is prevented, one may go with impunity among cholera patients.

The bacillus does not grow in acids, consequently when the digestion is active, the chances of taking cholera are small. It is only at the times when the stomach has ceased to act, as during attacks of indigestion from whatever cause, that cholera comes on. Persons in good health, who have firm nerves and lead correct lives, suffer little from cholera. The timid, the weak, and the dissipated are its principal victims.

The bacillus grows freely in water and on damp surfaces, consequently during an epidemic, raw fruit and vegetables should be avoided, and everything that is eaten should be freshly cooked.

The bacillus forms no spores, and is not found in the blood, consequently inoculation is not only useless, but positively dangerous, from the liability of introducing noxious substances into the blood.

The germ is easily killed. A ten per cent. solution of carbolic acid for twenty-four hours will kill the germs on any article of clothing. A solution of corrosive sublimate will do the same thing in a few minutes if the garment is thoroughly wet with it. Superheated steam for half an hour will do it, perhaps, the best of all. If a cover glass containing the bacilli be allowed to dry for three hours, they cannot be again revived; consequently, even drying may kill them. Fumigations are of doubtful utility. Cold checks their growth, but does not kill them.

We may hope, then, to stop every epidemic with the first case, if the diagnosis is made early, and the patient is isolated, and thorough disinfection practiced.